THE REACTION OF THE ERYTHROCYTES OF VERTEBRATES, ESPECIALLY FISHES, TO VITAL DYES

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In the erythrocytes of most vertebrates, but especially of fishes and amphibians, discrete granules are characteristically present. In the urodele, *Necturus,* bipolar clusters of such granules are regularly found in mature red blood cells. They are visible in fresh preparations, demonstrable as basophilic bodies with Wright or Giemsa staining, are blackened with osmic acid and silver salts, and are stained with iron hæmatoxylin after Helly fixation. Accordingly, there is no doubt that they are preëxistent structures and are not induced in supravital preparations by the action of the dyes.

However, secondary granules may also appear in such cells; the concentration of the dye, age of the preparation, brilliancy of illumination, and increase in temperature being effective as formative factors, influencing the rate and manner of their appearance (Dawson, 1928, 1929, 1930). Moreover, with higher concentrations of dye, the red cells may also exhibit elaborate patterns of reticulation. The genetic relation of both types of granules, preëxisting and induced, to the reticulated substance is an unsettled question. Morphologically there is no distinction between the two types and both are frequently enclosed in the reticular filaments. The reticulation pattern is apparently derived, through a reaction with the vital dye, from the basophilic substance which occurs diffusely in erythrocytes, and secondary granules are regarded by some as the same substance in a different form.

The literature on this subject is voluminous and many different views are advanced regarding the significance of the protoplasmic constituents reacting with vital dyes. First of all, the ability to react with a given vital dye may not be a specific characteristic of a single substance within the cytoplasm. Again, in many instances, the materials reacting with vital dyes are not always readily demonstrated by other technical methods. *Necturus* erythrocytes appear to be exceptionally favorable in this respect. Moreover, the amount of material reacting with vital dyes appears to decrease gradually as the erythrocytes differentiate, but the acquisition of hemoglobin in a given species proceeds only up to a certain stage at which the cell is said to be mature. Since the erythrocytes in different vertebrates do not attain at maturity the same relative degree

of differentiation, the picture in supravital preparations is subject to great variation; the individual changes which occur in the maturation of the red blood cells may apparently proceed at different rates and to different degrees in different species. Accordingly, the relative concentration of hemoglobin within the erythrocytes of different species appears to be to some extent independent of the amount and distribution of material reacting with vital dyes. These are only some of the factors which complicate the picture and make generalizations almost impossible. Coupled with these there is also the lack of complete information about the stages of maturation of these cells in many species. In other cases, too, findings by one method have not been adequately checked and confirmed by other technics.

Various theories of the origin and nature of the vitally-stained bodies have been advanced. Several earlier workers suggested a nuclear or nucleolar origin (Giglio-Tos, 1896; Jolly, 1903; Sabrazès et Muratet, 1908), while others have apparently confused them with centrosomes (von Apáthy, 1897; Bremer, 1895; Dehler, 1895; Eisen, 1897–1899; Golgi, 1920) when demonstrated by non-vital staining methods such as silver nitrate impregnation or staining with iron hæmatoxylin. They were also identified as intracellular parasites, but this view was soon dropped (Sabrazès et Muratet, 1900).

At present the vital granules are generally regarded as of cytoplasmic origin. Giglio-Tos (1896) suggested that they represented hemoglobin-forming substances and Yoffey (1929) is inclined to favor this view. Several modern investigators (Ferrari, 1930; Villa, 1930; Knoll, 1931) have advanced evidence in favor of an intranuclear origin of hemoglobin, and this interpretation accordingly would support the old hypothesis of Giglio-Tos that vital granules arise as nuclear emissions and are concerned in the elaboration of hemoglobin. On the basis of the reaction of these bodies with silver salts and osmic acid, they have been regarded as possibly homologous with the Golgi apparatus—dictyosomes (Bhattacharya and Brambell, 1925; Dawson, 1928, 1930; Dornesco and Steopoe, 1930*a*, 1930*b*; Urtubey, 1927). Nittis (1930) described a surface granule which in his opinion might be interpreted as the point of separation of two daughter cells or as a part of a trophospongial system. Nittis' view seems untenable, as the single granule is intracellular and may frequently exhibit Brownian motion (Dawson, 1931). Many tend to class all vital granules as artifacts (Chlopin, 1927; Weidenreich, 1903), failing to distinguish sharply between preëxistent and induced bodies (Beams, 1930).

The number and distribution of vital granules in the erythrocytes of vertebrates vary during the differentiation of the cell. Usually they are

not present in very young cells and are reduced or even disappear in old cells. In the intermediate stages they are frequently numerous and conspicuous. In the mature cells of different vertebrates in which they still persist, vital granules frequently occur in rather definite numbers and have a characteristic position within the cell. The more common types of distribution are single unipolar; multiple, clustered unipolar; multiple, clustered bipolar; multiple perinuclear; and multiple scattered or diffuse (Dawson and Charipper, 1929; Dawson, 1930, 1931).

The number and distribution of vital granules in the erythrocytes of cyclostomes and fishes vary considerably. They were apparently recognized as early as 1896 by Giglio-Tos, who observed them in the blood cells of the lamprey and called attention to the striking Brownian movements they exhibited. A few years later Sabrazès and Muratet (1900) described "corpuscles mobiles" within the erythrocytes of *Hippocampus*. They were irregularly distributed, with usually five to ten granules within a cell.

In the same year these investigators extended their observations to several more fishes. In *Torpedo oculata* the granules were numerous and distributed diffusely around the nucleus. The cells usually contained as many as forty granules although some had as few as three, four, and five. All granules exhibited Brownian movement. They were unequal in size; some were coupled and some elongated or compressed. In *Raia pastinaca* the bodies were frequently oval and relatively large but were not so numerous as in *Torpedo*. The blood cells of *Syngnathus typhle* apparently did not contain any granules. In *Petromizon marinus* and *Alosa finta* the granules were present but not numerous. In adult *Anguilla vulgaris* they were absent. In 1902, however, Sabrazès and Muratet found opportunity to examine the blood of some young eels, 6 to 7 cm. long. In these, vital granules with limited Brownian movement were present in about twenty per cent of the erythrocytes. They varied greatly in size, with usually one to three present in any one cell. In *Torpedo marmorata* (Sabrazès et Muratet, 1908) the number and distribution of vital granules is very similar to *Torpedo oculata*.

Jokl (1925) made an intensive study of the erythrocytes of *Raia clavata* and *Raia batis* and found in the main that the red blood cells were very like those of *Torpedo* in the number, size, and distribution of the vital granules. Further observations on the elasmobranchs were made by Lewis and Lewis (1926), who described numerous diffusely scattered perinuclear bodies in the dogfish and skate (*Raia erinacea*). These workers also described one to three neutral red granules in the erythrocytes of the sculpin, but illustrated the erythrocytes of the hake

and cunner as lacking any granular inclusions. Stolz (1928) found that the erythrocytes of *Cyprinus carpio* contained numerous granules diffusely distributed about the nucleus. Yoffey (1929) confirmed the observations of Jokl (1925) on the red blood cells of *Raia clavata* and *Raia batis*, and noted numerous fine basophilic granules in the mature cells of *Trigla gurnardus*. Dornesco and Steopoe (1930*a*) found that the erythrocytes of the dogfish are typical of the elasmobranchs in possessing numerous, scattered, actively motile granules. They (1930*b*) also investigated the blood of several marine teleosts, *Syngnathus acus*, *Blennius pholis*, *Solea vulgaris*, *Pleuronectes platessa*, *Gobius paganellus*, *Cottus bubalis*, *Labrus melops*, *Onos mustella* and *Nerophis lumbriciformis*. In all of these they found that the erythrocytes contained uniformly one granule, usually located eccentrically near one pole of the nucleus. The conditions in the erythrocytes of *Ameiurus nebulosus* are the same as in the marine teleosts (Dawson, 1931).

In the erythrocytes of the fishes studied the distribution of vital granules is not so variable as in the Amphibia, being limited chiefly to two types, single unipolar and multiple perinuclear, restricted or diffuse. The characteristic unipolar and bipolar clusters appearing in the red blood cells of many urodeles are not found. It has seemed advisable to extend the observations on the blood of fishes, with a view to obtaining more information regarding the relationships between the preëxistent and induced granules and the reticular substance as revealed by vital dyes.

Material and Methods

During the summer of 1931 the blood of seventeen different species of fish, taken in the vicinity of Woods Hole, was examined. Supravital staining was carried out by the dry dye-film method, using neutral red alone, neutral red in combination with Janus green, and brilliant cresyl blue. The concentration of neutral red, 1 : 1250, previously used on amphibian blood proved satisfactory in the case of fishes, but more Janus green had to be added to obtain a clear view of the chondriosomes. Films of brilliant cresyl blue were made with a saturated solution in absolute alcohol. Permanent preparations of supravitally stained blood were made by the method of Scott (1928). Smears stained by Wright's method were also prepared. In all cases the blood was freshly drawn from the heart of living fish.

It is essential that neutral red alone be used to check neutral red-Janus green B preparations, since the toxicity of Janus green may cause injuries which result in more rapid induction of bodies stainable with neutral red. On the other hand, Janus green B cannot safely be used alone, since bodies regularly stained with neutral red may take up Janus

green and confuse the chondriosome picture. However, when the dyes are used in combination, the neutral red displays a greater affinity for the bodies which are regularly stained by it and the Janus green reaction is accordingly confined entirely to the chondriosomes. The brilliant cresyl blue was applied at such high concentrations that the nuclei were stained and the reticular patterns produced almost immediately, and there did not appear to be any progressive induction of formed bodies (granules) while the preparations were being studied for reticulation patterns.

Description

Before proceeding to the description of the reactions of the erythrocytes of the different fishes to vital dyes, certain general features of these reactions will be discussed. In most fishes there are sufficient immature red cells, in varying stages of differentiation, that the pictures presented after supravital staining lack uniformity. This makes the problem of interpretation more difficult, since the differences in hemoglobin concentration in some cases are scarcely perceptible. Differences in the degree of maturity of such cells are most strikingly demonstrated with brilliant cresyl blue and the reticulation patterns furnish an excellent index of cell age. The presence of young red cells in the blood is readily confirmed in smears differentially stained by Wright's method, where varying degrees of basophilia and polychromasia are clearly demonstrated.

As the erythrocytes mature the amount of material reacting with brilliant cresyl blue gradually decreases and the patterns of reticulation also undergo changes in form and distribution. In very young cells the entire cytoplasm is filled with fine, densely massed granules with little evidence of real reticulation. In succeeding stages of differentiation the pattern assumes a true reticular form. At first the meshes are small and distributed throughout the whole cell. Later the meshes are more open and the reticulation does not extend completely to the periphery of the cell, assuming the form of a perinuclear wreath. As the cells grow older, the meshes are still wider and the filaments eventually are partially interrupted, forming an open, fragmented wreath. As the reticular substance is further reduced, the filaments of the net are more frequently interrupted and the wreath-like form as well as the reticular appearance is lost. The more or less separated filaments then appear radially arranged about the nucleus, with very fine fragments interspersed between them. With the further disappearance of reticular substance the pattern is reduced to scattered, irregular, short filaments and fine, dust-like granules. In final stages the filamentous form may practically disappear, leaving a variable number of fine granules.

It is difficult to determine whether the reticular substance, which is demonstrated with concentrations of the vital dye sufficiently high to stain the nuclei immediately, is of the same constitution as the relatively large granules which may be induced in similar blood cells by a prolonged exposure to the same dye in a lesser concentration, which will not stain the nucleus or produce reticulation. At present, it seems impossible to decide this question. On the other hand, it is frequently possible to distinguish clearly between preëxistent vital granules and the induction patterns, since in many cases the former may be seen in fresh untreated cells or may be demonstrated in fixed cells by a variety of reliable technical methods.

With high concentrations of brilliant cresyl blue, nuclei, nucleoli (when present), preëxistent and induced granules, and reticular substance are all stained. The nuclei are pale blue, nucleoli deep blue, all granules deep blue-purple and the reticular substance a red-violet. The nucleoli, varying in number from one to three, are large and conspicuous in the erythroblasts but become progressively smaller as the cells mature. They may be distinguished as minute bodies in cells of the "wreath" stage but seldom persist in more mature forms. The difference in the color reaction of the granules and the reticular substance may or may not be significant. The granules are sharply limited, dense, and highly refractile, while the reticular substance has an irregular appearance as if formed by the aggregation of very fine particles. The difference in physical state accordingly may explain the different shades presented by the two types of bodies.

The form and distribution of chondriosomes within the erythrocytes of the fishes vary in the different stages of maturity, but are nevertheless quite characteristic for the type of cell. In the younger stages they are quite numerous, granular, and diffusely scattered throughout the cytoplasm. In later stages the number is greatly reduced and they are more closely aggregated about the nucleus. Their form, too, may be changed, many appearing as long, tortuous bodies. In fully mature cells the chondriosomes are usually entirely of the filamentous type, although occasional granular forms are seen. Some of these are not true granules but represent filaments oriented lengthwise between the two flattened surfaces of the erythrocyte and may be observed to shift in position as the cell is modified by the injurious effects of the Janus green.

It is difficult to obtain sharp images of chondriosomes in the fresh cell, and relatively high concentrations of dye must be used. As the staining progresses the chondriosomes are first seen as hazy blue-green bodies, and at this time the nuclear outline is also indistinct. Shortly both the nuclear membrane and chondriosomes are distinctly seen and

the cytoplasm of the cell appears clearer. While these changes are taking place striking movements of the mitochondria may be observed. Filaments which were closely applied to the nucleus may frequently swing out to lie at right angles to the nuclear surface or may move completely away, and others which were seen on end may assume a position parallel with the flattened surface of the cell.

For purposes of description the fishes studied at Woods Hole will be divided into two major groups, based on the distribution of the primary or preëxistent granules as demonstrated by low concentrations of neutral red. In the first group the vitally stained granules are usually single, with occasionally one or more accessory bodies, and are located eccentrically near one pole of the nucleus. In the second group the granules are numerous and may either have a definite perinuclear arrangement or be scattered more or less diffusely throughout the cytoplasm. The reaction of the primary granules to Wright's stain might also be used as a basis of classification, since in some fishes the granules uniformly give a basophilic reaction; in others this reaction is limited to a varying number of cells; while in still others the granules always remain unstained. The three second groups would form natural subdivisions of the major Group I, which is based primarily on the distribution of granules, but in Group II this would not hold true since in no instances do the perinuclear or diffusely arranged granules react uniformly with Wright's stain.

Group I

Toadfish, *Opsanus tau* (Linnæus). With neutral red the primary granules ordinarily appear as single, unipolar bodies, although one or more accessory granules are often encountered (Fig. 1, *b* and *c*). The accessory granules are usually small, but in some cases all the granules are of equal size, resembling the variations described for *Ameiurus* (Dawson, 1931). After long exposure to neutral red the granules are increased in number and tend to form clusters about the primary bodies. Later, other secondary granules appear irregularly throughout the cell.

The mitochondria are relatively few, varying in number from three to six. They usually appear as wavy filaments but are frequently dilated to encapsulate a large spherical refractile body. These bodies are readily seen after the Janus green has been reduced. They do not take up neutral red and their significance is not known.

Very little reticular substance is demonstrated by brilliant cresyl blue. The filaments are short and are usually radially arranged about the nucleus. Some end in contact with the cell membrane. Primary granules and a variable number of secondary granules are stained with

this dye. In the cells with the least reticulation practically no induction of granules has taken place (Fig. 1, *d*). The cells with the most reticulation contain many more granules (Fig. 1, *a*). All the cells appear relatively mature. An occasional cell in the "wreath" stage with a clear border was encountered, but there were no younger stages.

In smears stained by Wright's method the primary granules appear as distinct basophilic bodies. No basophilic erythrocytes are present and only a few cells exhibit polychromasia.

Tautog, *Tautoga onitis* (Linnæus). The neutral red patterns are quite similar to those of the toadfish (Fig. 4, *a* and *b*). A number of erythrocytes possess granular mitochondria, but in the majority of the cells they are filamentous. The reticular substance is scanty in most cells (Fig. 4, *d*), but a few "wreath" stages were seen (Fig. 4, *c*). Occasional small cells (erythroblasts) with a dense reticulation and a conspicuous nucleolus are present.

With Wright's stain the primary granules are stained blue and a few erythrocytes show basophilia and polychromasia. The number of immature red cells is, however, very small.

Cunner, *Tautogolabrus adspersus* (Walbaum). The erythrocytes of this fish very closely resemble in their staining reactions those of the two preceding forms (Fig. 6, *a* and *b*). Fewer immature cells were noted than in the tautog, and in general the reticulation patterns are very meager (Fig. 6, *c* and *d*). The primary granules also appear as basophilic bodies with Wright's stain.

Sea Bass, *Centropristes striatus* (Linnæus). The primary neutral red bodies are the same as in the erythrocytes of the preceding fishes (Fig. 3, *a*). Induction of secondary granules occurs rather freely (Fig. 3, *b*), but even when the secondary staining effect has appeared there are a number of apparently mature cells which do not show any reaction to the dye. The mitochondria are relatively few, the filamentous form predominating.

With brilliant cresyl blue many cells fail to show any reticular patterns but contain only granules such as are seen after moderate induction with neutral red. Other cells contain a variable number of reticular filaments (Fig. 3, *c*), but the "wreath" stage was observed in only a few cells (Fig. 3, *d*). Occasional young cells with a fine, dense reticulum and conspicuous nucleoli were encountered.

In the sea bass virtually all the erythrocytes stain orthochromatically with the eosin in Wright's stain. Polychromasia is rarely seen and occasional round basophilic erythroblasts are present. The primary granules demonstrated so readily with neutral red are not usually stained in the smears. Some of the larger granules appear as distinct baso-

philic bodies, other smaller granules are barely distinguishable, and many cells appear not to have any granular inclusions.

Sea Robin, *Prionotus carolinus* (Linnæus). The erythrocytes of the sea robin in their staining reactions are very like those of the sea bass (Fig. 2). The mitochondria, however, usually appear as short rods and granules rather than filaments. The reticular patterns are very sparse, being represented by scattered particles radially aligned about the nucleus (Fig. 2, *d*). All the cells appear to be mature. This conclusion is supported by the staining reactions of the erythrocytes with Wright's stain. No granular inclusions could be distinguished in the stained smears.

Scup, *Stenotomus chrysops* (Linnæus). The neutral red patterns and mitochondria present no unusual features (Fig. 7, *a* and *c*). The reticular patterns are quite variable, although in the majority of cells the filaments are reduced to a minimum (Fig. 7, *b*). Young cells with a dense reticulum and conspicuous nucleoli are frequently seen and stages with fragmented open-meshed "wreaths" are quite numerous (Fig. 7, *d*). The presence of such immature cells is confirmed by an examination of stained smears, but the preëxistent or primary granules were not demonstrable as basophilic bodies.

Butterfish, *Poronotus triacanthus* (Peck). The primary granule is characteristically present in neutral red preparations (Fig. 8, *b*) and induction of new granules proceeds very slowly. The chondriosomes are usually granular. No long filamentous forms were observed. The reticular patterns and numbers of immature cells are about the same as in the scup (Fig. 8, *a*, *c*, and *d*).

Variegated minnow, *Cyprinodon variegatus* Lacépède. The primary neutral red bodies usually appear as single or double bodies and induction of secondary bodies occurs slowly. The mitochondria are predominantly of the granular type and are relatively numerous (Fig. 11, *a* and *b*). In the majority of the cells the reticular material is very scanty (Fig. 11, *c* and *d*), but in a few cells complete perinuclear "wreaths" of reticular material were seen. Occasional younger cells, possessing large nucleoli and dense reticulations, were also noted. The primary neutral red bodies of mature cells did not give a basophilic reaction with Wright's stain, but in many polychromatic cells they could be distinctly seen as basophilic granules.

Mummichogs, *Fundulus heteroclitus* (Linnæus) and *F. majalis* (Walbaum). The behavior of the erythrocytes is essentially alike in these two species. The primary neutral red bodies are characteristically present (Fig. 12, *b* and *c*). The reticular substance in general is scanty, appearing as radially arranged filaments (Fig. 12, *d*). A few

cells in the " wreath " stage and an occasional young cell with a large nucleolus were seen. The primary granules were not demonstrated by Wright's method.

Common eel, *Anguilla rostrata* (Le Sueur). The neutral red bodies are very minute, almost at the limit of visibility (Fig. 5, *a* and *d*). However, they gradually increase in size on exposure to the dye and secondary granules slowly appear (Fig. 5, *b*). The mitochondria are few in number and chiefly of the long, sinuous type. Brilliant cresyl blue reveals the presence of large numbers of immature erythrocytes, the reticular substance varying in distribution from complete " wreaths " to scattered isolated filaments and granules (Fig. 5, *c*). In stained smears many erythrocytes show varying degrees of cytoplasmic basophilia. The primary granules frequently appear as basophilic bodies in cells which stain orthochromatically in eosin or show a slight polychromasia. However, they were not distinguishable in more basophilic cells.

Sand dab, *Hippoglossoides platessoides* (Fabricius). The primary vital granules are readily demonstrated as single or double granules (Fig. 9, *a*). With longer exposure to the dye secondary granules, usually grouped in clusters, quickly appear (Fig. 9, *c* and *d*). Chondriosomes of both filamentous and granular types are present. Practically all the cells are mature, showing a very sparse reticulation (Fig. 9, *b*). An occasional cell with an incomplete open-meshed " wreath " was seen. A few basophilic erythrocytes were demonstrated with Wright's stain. The primary granules were frequently seen as basophilic bodies in erythrocytes which exhibit a slight polychromasia but could not be distinguished in mature cells.

Group II

In the erythrocytes of the fishes of this group the primary granules are not limited to a single or double unipolar body but are relatively numerous and have a perinuclear distribution.

Menhaden, *Brevoortia tyrannus* (Latrobe). In the menhaden the granules are very small, frequently barely visible at a magnification of nine hundred diameters. The granules are either arranged in a single definite line about the nucleus or scattered somewhat irregularly throughout the cytoplasm (Fig. 10, *a* and *b*). The irregular distribution of granules appears to be characteristic of the oldest cells. On longer staining with neutral red they increase in both size and number. Mitochondria of both the filamentous and granular types are present in restricted numbers.

All stages of maturating erythrocytes were encountered, but the majority of the cells were fully differentiated. The reticular patterns

vary all the way from a dense compact granular mass filling the entire cell to the mature condition in which only fine particles and scattered, short filaments are present (Fig. 10, *c* and *d*). In stained smears erythrocytes in the different stages of differentiation are clearly shown and the numerous perinuclear primary granules are seen distinctly as basophilic bodies in both polychromatic and mature cells.

Alewife, *Pomolobus pseudoharengus* (Wilson). The erythrocytes of the alewife are essentially like those of the menhaden in all their reactions to the dyes, but more immature cells are present (Fig. 14). Many of the younger cells contain large, clear, refractile globules which appeared as vacuoles in stained smears.

Mackerel, *Scomber scombrus* Linnæus. The erythrocytes of the mackerel differ only slightly from those of the menhaden and alewife (Fig. 13). The primary neutral red patterns are alike. Mitochondria of the filamentous type predominate. More immature cells are present than in the alewife and the reticulation patterns are accordingly very variable. The red cells react rapidly with neutral red and many secondary granules may develop. The primary neutral red granules are demonstrated as basophilic bodies by Wright's stain, but much more care must be taken in the differentiation of the stain.

Smooth dogfish, *Mustelus canis* (Mitchill) and spotted skate, *Raja diaphanes* Mitchill. The vital staining reactions of the erythrocytes of both these forms have been described by several investigators. The granules appearing after exposure to neutral red are very large and numerous (Figs. 15 and 17). The mitochondria are usually long and filamentous. Induction of new granules occurs rapidly. There is a relatively high proportion of immature red cells, and the reticulation patterns are very variable. In old cells the reticular substance is greatly reduced, appearing as scattered particles and short filaments.

With brilliant cresyl blue both vital granules and reticulation patterns are demonstrated as in the teleosts, but on long standing the dye in the granules disappears while the reticular substance remains brilliantly stained. The reduction of the dye in the granules appears to be characteristic only of the elasmobranchs and has been previously reported by Jokl (1925). Although the granules are large and readily seen in fresh unstained preparations, they cannot be demonstrated in smears stained by Wright's method and do not give a basophilic reaction.

The Relative Degree of Differentiation of the Mature Erythrocytes of Vertebrates

In the running description of the findings in the erythrocytes of the different species of fish, the staining reactions described related particu-

larly to the predominating cells, presumably the mature ones. During the course of the study it became increasingly obvious that no granules, either preëxistent or induced, were present in young cells exhibiting a complete, dense reticulation pattern. In somewhat older stages the primary granules were readily demonstrated, but induction of new granules either did not occur or proceeded very slowly. This secondary reaction varied greatly in different species, but the general trend was the same in all. In more mature stages induction usually occurred more readily, but in some old, perhaps senile, cells, which gave practically no reticulation reaction with brilliant cresyl blue, the secondary response to vital dyes again decreased.

The terms "primary" or "preëxistent," and "secondary" or "induced" are used here without reservation to designate the granules which are under discussion, since the cumulative evidence gathered from studies on fishes and amphibians indicates that such a distinction is valid. The granules which stain so readily with neutral red are frequently seen as refractile bodies in fresh preparations, and in dry-fixed smears are frequently demonstrated as basophilic inclusions. In most cases the characteristic form and location of these elements make their identification easy when they are rendered visible by other than supravital methods. Dornesco and Steopoe (1930*a*, 1930*b*) have also successfully blackened these primary bodies with silver nitrate methods (Da Fano and Cajal) in both teleosts and elasmobranchs. Their demonstration by a silver method in the elasmobranch erythrocyte is significant, since in these cells the granules fail to give a basophilic reaction in carefully stained smears.

The reality of the secondary granules cannot be disputed since they can be observed as they arise within the cells. Accordingly we have to deal with three distinct morphological entities, at least as they are demonstrated by supravital methods. The question of their chemical constitution and relationships is a baffling one and apparently little further progress can be made in this regard until better methods of study are devised.

Nevertheless, the characteristic reactions to vital dyes may be legitimately used as evidences of the progressive changes which occur within the differentiating erythrocyte, and such reactions therefore constitute useful criteria for determining the degree of differentiation reached by the mature erythrocytes of a given species. The criteria of maturity in one species are not necessarily valid in every detail for erythrocytes of another species, since the pictures obtained by vital dyes may vary in different groups of animals. Maturity of erythrocytes in general can best be defined as the stage at which the cell acquires its maximum

concentration of hemoglobin, and in normal animals cells of this type should predominate in the circulation. The degree of concentration of hemoglobin in mature cells in a given species is relatively constant and is closely correlated with the acquisition of characteristic staining reactions, but similarity of staining patterns in different species does not by any means indicate that the different erythrocytes have acquired the same concentration of hemoglobin. In other words, the erythrocytes of the different vertebrates are mature at varying levels of differentiation, the latter being measured by such staining reactions as are usually regarded as evidences of immaturity in the most highly differentiated red cells.

During the differentiation of the vertebrate erythroblast a striking series of changes occurs. Some are readily demonstrated in fixed preparations while others are adequately revealed only by supravital staining. Most of these changes are common to the erythrocytes of all vertebrates, but in the mammals an extreme degree of specialization is encountered. During differentiation the nuclear-cytoplasmic ratio changes greatly, the nucleoli undergo a gradual involution and may disappear, the distribution of chromatin in the nucleus is modified, and the basophilia of the cytoplasm is gradually lost and replaced by the eosinophilia of the hemoglobin. Also the volume of mitochondrial substance is progressively reduced, and frequently the form of the individual elements is changed.

The gradual reduction of the reticular substance, definitely correlated with a decreasing basophilia of the cytoplasm, is the most striking feature of the maturing erythrocytes when seen in supravital preparations. Less conspicuous changes involve the appearance and behavior of both types of granular inclusions, preëxistent and induced. Primary granules are usually absent from very young cells and disappear in a later but somewhat variable stage of differentiation. When they first appear they do not give a basophilic reaction in stained smears, but later in many instances they are characteristically basophilic. In mature cells of some animals the basophilic reaction is subsequently lost and the bodies are again demonstrated only by supravital methods. Moreover, in some vertebrates the primary granules may entirely disappear while in others they persist and assume characteristic patterns of distribution. Other granules of similar morphology and behavior may appear in cells that have stood in preparations for some time. Apparently induction of granules can occur only after some degree of differentiation of the erythrocyte has been attained, but the ability to respond in this manner may persist until full maturity is reached, and in many instances induced granules may appear even after the primary granules are no longer in evidence. In the mammals, however, the nucleus and all the cytoplasmic inclusions eventually disappear completely.

In the different vertebrates each of these changes in the maturing erythrocyte may take place at a different rate and to a varying degree. Accordingly, with the exception of the mammals, it is not always easy to decide which cells represent at maturity the more fully differentiated stage. In all vertebrates below the mammals the cells are characteristically nucleated with a few striking exceptions in the Amphibia (Emmel, 1924). In all of these animals the basophilia is eventually replaced by eosinophilia. The nucleoli may disappear; the nuclear-cytoplasmic ratio undergoes considerable changes, the nucleus becoming condensed and acquiring a characteristic chromatic pattern. That is, in ordinary stained smears the mature nucleated erythrocytes of vertebrates appear essentially alike except that they vary greatly in size. However, with the more delicate methods of supravital staining, quite striking differences are brought out. Accordingly, the patterns of granulation and reticulation afford the best criteria of the degree of differentiation of these cells, and of these two criteria the degree of persistent reticulation is probably the better since the reticular material is continuously present in the cell while the granules have a variable and much more complicated history. Still, the latter criterion cannot be entirely disregarded in comparing the nucleated red cells of the vertebrates.

Adequate data for the comparison of the erythrocytes of vertebrates are available for fishes and amphibians, but the blood of reptiles and birds has not been studied so extensively by means of vital dyes. In order to secure first-hand information concerning the conditions in the reptiles and birds, supravital preparations of blood from the painted turtle, horned toad, fence lizard (*Sceloporus undulatus*), and the common fowl were studied.

In the fishes (both elasmobranchs and teleosts) and birds little reticular substance is present in the mature cells (Fig. 18, *a*, *c*, and *d*), being represented mostly by scattered filaments and granular fragments. My observations on the fowl differ from those of Doan, Cunningham, and Sabin (1925), who report that the final stages of reticulation consist in a few discrete bodies stainable with vital dyes. These bodies are readily demonstrated by low concentrations of either neutral red or brilliant cresyl blue (Fig. 18, *b*), but when brilliant cresyl blue in sufficiently high concentration to stain the nuclear reticulum is used, scattered fragments and short filaments are also uniformly present in the cytoplasm. Brilliant cresyl blue is apparently reduced to some extent in the cell and the demonstration of persisting fragments of reticulum is possible only when the dye is present in considerable excess. In the amphibians and reptiles, on the other hand, a definite, more or less complete reticular pattern can be demonstrated in the mature erythrocytes, but in most

instances the amphibian erythrocytes contain the greater amount of reticular substance (Figs. 16, 19, 20, and 22). If the degree of persistence of reticulation is regarded as evidence of the degree of differentiation attained by the mature erythrocyte, it must be concluded that the red blood cells of fishes and birds are relatively more highly differentiated than those of the amphibians and reptiles.

The amount of material demonstrable as so-called primary vital granules in the mature erythrocytes of the several classes of vertebrates cannot be readily correlated with the degree of persistent reticular substance, but the history of the vital granules indicates that their presence in red blood cells is in some degree a measure of relative maturity. It has been already pointed out that the reticular substance is at a maximum in the young cells and gradually decreases as the erythrocytes mature, while in general the granular substance is not present at all until the cells are partially mature.

It is practically impossible to make any significant generalizations regarding the vital granules. The erythrocytes of each species must be considered separately if any accurate conclusions are to be drawn regarding relative maturity of the cells. It has been held by many that the granular material is but a phase of the filamentous reticulum. In very young cells the reticular substance is definitely granular before acquiring the filamentous form. The granular form is doubtless dependent on its concentration within the cell and is the result of the agglutinating or precipitating effect of the vital dye. The primary granules are, however, definite, discrete bodies, frequently distinguishable in fresh unstained cells, and may often be demonstrated in fixed material. They are apparently associated in some way with cell metabolism, and when the erythrocytes are exposed to a penetrating dye it accumulates first of all in these preformed structures. Whether the secondary or induced bodies are derived from reticular material is less easy to determine. But certain lines of evidence would appear to indicate that they are the result of a specific reaction of the cytoplasm to an excess of dye and do not represent local, sharply delimited accumulations of reticular material (Chlopin, 1927).

The history of the behavior of these three elements in the maturing erythrocytes further suggests that they are separate entities. In elasmobranchs the primary granules of mature cells are large and numerous when the reticular substance is reduced to the same degree as in the teleosts. In the amphibians the history is also variable, granules being either present or absent, depending on the species (Arrigoni, 1908; Beams, 1930; Dawson and Charipper, 1929; DeRoo and Ufford, 1930; Goda, 1929; Hibbard, 1928; Jordan, 1925; Stolz, 1928), while the

reticular material persists as a fairly complete, open network. In *Triturus viridescens* the erythrocytes rarely contain any granules either primary or secondary, but as Nigrelli (1929) has shown, the reticulation in mature cells is relatively abundant (Fig. 16). Other similar examples could be cited.

In the reptiles the reticulation is quite abundant in mature cells but the primary granules are single in the alligator, horned toad, and fence lizard, and clustered at one pole in the painted turtle. The granules in these several species can also be demonstrated as basophilic bodies in dried smears stained by Wright's method (author's observations), and the question of their being induced bodies cannot be raised. In the common fowl and pigeon, Doan, Cunningham, and Sabin (1925) regard the single vital granule as a vestige of the reticular substance. Forkner (1929) also states that in the domestic fowl the cytoplasm of the erythrocytes contains, after staining with neutral red, from none to several small, reddish brown bodies which are usually near the nucleus but often move about and may be far out near the cell border. These bodies are not demonstrated in smears stained by Wright's method but, as pointed out earlier, they have probably been demonstrated by other methods and mistaken for centrosomes. Their close resemblance to similar structures in teleosts and reptiles which can be shown to be primary bodies lends strength to the view that they are preëxistent in the fowl erythrocytes, and are not produced by a reaction of the dye with remnants of the reticular substance. In view of the evidence accumulated from a study of the nucleated erythrocytes of vertebrates, the author is inclined to accept the conclusion of Michels (1931) that the reticular substance in reticulocytes has no genetic relation to the vital granules of the mature red cells, but would disagree with his acceptance of the view that vital granules are surface structures, either precipitates of the stain or stained precipitates of the plasma. After a study of the irregular behavior of the vital granules in nucleated erythrocytes, it does not seem surprising that such granular cytoplasmic inclusions do persist even after the nucleus, chondriosomes, and reticular substance have disappeared from the mammalian cell. The appearance of primary granules in the cytoplasm is apparently a constant phenomenon in the maturation of the erythrocytes. It is only in the mammalian erythroplastid that they uniformly completely disappear and in this instance they mark the acme of erythrocyte differentiation. They, however, are not always the last vestige of immaturity to disappear, since in some higher urodeles and several anurans they disappear while the reticular substance is still present in relatively large amounts.

The appearance of secondary or induced granules in erythrocytes

following exposure to vital dyes apparently has only a very limited relation to the degree of differentiation attained by such cells. It is true that young cells with a high concentration of reticular substance react slowly and to a very limited degree to dyes, but the amount of reaction obtained in more mature cells is also very variable and seems not to be directly determined by the degree of differentiation attained. Rather the reaction appears to be species specific and to depend on the permeability of the cell and other factors inherent in the cytoplasm of the given species. Also the degree of the reaction with the cytoplasm cannot be correlated with the presence of a certain amount of reticular substance, since as much induction may occur in mature erythrocytes of fishes with a minimum of reticulation as in those of amphibians and reptiles where the reticular substance persists in greater amounts. The induction phenomenon in cells containing primary granules is usually confined at first to the areas of the cell containing such preëxistent bodies. In the early stages of induction the primary granules themselves become enlarged and new granules then appear in their immediate vicinity. Later new bodies may form irregularly throughout the cytoplasm.

The phenomenon of induction in erythrocytes appears to be closely related to the "krinome" reaction to vital dyes described by Chlopin (1927) for many other cells of the animal body. Freely penetrating stains such as the basic dyes appear to accumulate within the cytoplasm of the red blood cells and appear first in the preformed bodies when they are present. Later, as more dye is accumulated, it is segregated by some reaction of the cytoplasm into newly formed structures. In some erythrocytes this accumulation and subsequent segregation of dye within the cytoplasm appears to continue progressively and to surprising limits, while in other erythrocytes the reaction proceeds only to a minimum extent. As has been already noted, many of the external factors influencing this reaction are known, but the factors within the cytoplasm, which are apparently of utmost importance, are unknown. The reaction, as in the anurans (Beams, 1930), proceeds as well in mature cells without preformed bodies as in cells in which preformed bodies are numerous and conspicuous. The final loss of the ability to react, as in the most highly differentiated mammalian erythrocytes, would seem to indicate that the degree of differentiation attained by the cell in some way determined or limited the cytoplasmic reaction to the dye. Such a conclusion, however, is rendered more or less untenable by the irregular behavior of the nucleated erythrocytes of other vertebrates, whose degree of differentiation at maturity can be estimated by the degree of persistence of reticulation. In such cells, except in very young stages,

there is no significant correlation between the relative degree of differentiation attained and the degree of neo-formation of vitally stained bodies.

Before concluding this discussion of the relative degree of differentiation of the mature vertebrate erythrocytes, one other morphological feature of maturing erythrocytes should be mentioned, although the observations on it are not all comprehensive. It has already been noted that with relatively high concentrations of brilliant cresyl blue the nucleoli in immature cells of the fishes appeared as deep blue bodies in a light blue, apparently homogeneous nucleus, the nuclear reticulum not being shown with such concentrations of the dye. The nucleoli (plasmosomes) are relatively large in young cells and vary in number, but there are seldom more than three in any cell. They grow smaller as the erythroblasts differentiate and usually appear as single, small, spherical bodies. After the stage at which the reticular substance appears as an open-meshed, almost complete network the nucleoli are more rarely seen, and in the mature cells containing scattered fragments of reticulum they are usually absent. In the catfish, however, nucleoli are uniformly present in the mature erythrocytes.

Nucleoli are not readily demonstrated in the mature cells of the Amphibia by brilliant cresyl blue, but they may be stained if concentrations of dye sufficiently high to bring out the chromatin reticulum of the nuclei are used. The dye, however, must not be intense enough to stain the chromatin a dark blue or the nucleoli are obscured (Fig. 16). In such preparations of the erythrocytes of *Necturus* and *Triturus* the nucleoli, numbering from one to four, appear as dark blue bodies lying between the coarse bars of light blue chromatin. They are somewhat irregular in form and are not so sharply outlined as in the fishes. At first it seemed doubtful if these bodies were nucleoli, but a comparison of the mature cells with younger stages in the circulation appears to establish their plasmosomal nature.

In the reptiles studied (horned toad, fence lizard, and painted turtle) for this feature of the mature erythrocyte, the nucleolus is uniformly found as a single spherical body in all mature erythrocytes, and is a striking feature of all properly stained preparations (Figs. 19, 20, and 22). Immature cells were rarely encountered in the blood of these animals and no comparisons with the nucleoli of younger erythrocytes were made. In the blood of the fowl no nucleoli could be distinguished in mature cells; but in an occasional immature cell, still in the stage with a more or less complete reticular net, small single spherical nucleoli were observed.

This method of demonstrating nucleoli, supravitally with brilliant

cresyl blue, in red blood cells does not appear to have been previously employed. It seems to be a delicate method and to give clear pictures of nucleoli even when they cannot be easily demonstrated in fixed and stained preparations. In many amphibian erythrocytes brilliant cresyl blue also stains the achromatic contents of the nucleus a reddish violet, while the chromatin is a light blue and the nucleoli deep blue. In such cells the nucleoli appeared to be imbedded in the achromatic substance.

These observations on the persistence of the nucleoli are correlated in a very satisfactory manner with the findings on the degree of persistence of reticular material and afford additional evidence that the mature erythrocytes of fishes and birds are relatively more highly differentiated than those of amphibians and reptiles.

The degree of differentiation of erythrocytes of the several classes of vertebrates as determined by the criteria of persistent reticulation and presence of nucleoli also seems to correlate fairly well with the size of these cells. The Amphibia as a class have the largest erythrocytes (Fig. 21). Reptiles have blood cells next in size and fishes come next, then birds and mammals. It is difficult, however, to conceive that cell size could directly influence the degree of persistent reticulation or the persistence of nucleoli. The presence of primary granules might possibly be dependent to some extent on this factor, since the products of metabolism might be less readily eliminated from larger cells and temporary accumulations be segregated in the cytoplasm in the form of granules.

Summary

The reactions of the mature erythrocytes of seventeen species of fishes to the vital dyes neutral red, Janus green B, and brilliant cresyl blue, have been studied. In most teleosts the primary vital granules are readily demonstrated by neutral red and consist of one or two small granules eccentrically placed near one pole of the nucleus, but in the menhaden, alewife, and mackerel the primary granules are most numerous and are either arranged in a single definite line about the nucleus or scattered irregularly throughout the cytoplasm. In the elasmobranchs the granules are large, numerous, and scattered. In a majority of the teleosts the primary granules may be demonstrated as basophilic bodies in dry films stained by Wright's method and are also frequently seen in fresh unstained preparations.

Secondary or induced granules may also appear in the cytoplasm of cells exposed for long periods to the dye. The degree of induction of new bodies does not appear to depend entirely on external factors but is determined to a large extent by factors inherent in the cytoplasm of the

given species. In general the mitochondria are filamentous, reduced in number, and lie in close contact with the surface of the nucleus.

The reticular substance in all mature erythrocytes of the fishes is greatly reduced and appears either as short irregular filaments or as minute granular remnants. It is best demonstrated with brilliant cresyl blue.

An attempt is made to compare the relative degree of differentiation attained by the mature erythrocytes of the several classes of vertebrates. The following criteria have been considered: changes in nuclear-cytoplasmic ratio; chromatin distribution in the nucleus; involution of the nucleoli; loss of basophilia; changes in the form, distribution, and volume of mitochondrial substance; reduction of reticular substance; amount of primary granules; and degree of induction of secondary granules. Of these criteria the degree of persistence of reticulation has been found to be the most consistent, and on this basis the several classes of vertebrates are arranged in the following ascending order of relative differentiation attained by their erythrocytes at maturity: amphibians, reptiles, fishes, birds, and mammals. This arrangement is also supported by the behavior of the nucleoli, which persist in the erythrocytes of amphibians and reptiles but are not usually demonstrated in the mature cells of fishes and birds.

The history of the primary and secondary granules is less regular and consequently less useful for measuring the relative differentiation attained by the cells of different classes of vertebrates. However, within a given class of vertebrates it is concluded that the presence of a large number of primary granules or the rapid induction of new granules in mature cells may be regarded as supplementary evidence of a lesser degree of differentiation. The presence of primary granules or the degree of induction of new granules, however, cannot always be correlated with the degree of persistence of reticulation.

It is also concluded on the basis of this survey of the vertebrate erythrocyte that primary granules, secondary granules, and patterns of reticulation as revealed by vital dyes, must be regarded as three separate entities which are not genetically related.

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EXPLANATION OF PLATES

All drawings are from dry preparations and were outlined at the same magnification by means of a camera lucida. The details were filled in free-hand from sketches of the fresh cells. Erythrocytes stained with brilliant cresyl blue to demonstrate patterns of reticulation are shown with solid nuclei. All others, with the exception of Fig. 21, were stained either with neutral red and Janus green B or neutral red alone. The mitochondria are usually filamentous but some granular forms are present. Ordinarily they may be distinguished by their juxta-nuclear position.

Plate I

Explanation of Figures

1. Erythrocytes of the toadfish showing, (*a*) induction of granules and reticulation; (*b* and *c*) primary granules and mitochondria; (*d*) primary granules and reticulation.

2. Erythrocytes of the sea robin showing, (*a* and *b*) the primary granules and mitochondria; (*c*) induction of granules after twenty minutes; (*d*) reticulation.

3. Erythrocytes of the sea bass showing, (*a*) primary granules and mitochondria; (*b*) induction of granules after twenty minutes; (*c*) reticulation in a mature cell; (*d*) reticulation in an immature cell which possesses a nucleolus.

4. Erythrocytes of the tautog showing, (*a* and *b*) primary granules and mitochondria; (*c*) reticulation in an immature cell; (*d*) reticulation in a mature cell.

5. Erythrocytes of the eel showing, (*a* and *d*) primary granules and mitochondria; (*b*) induction of granules after twenty minutes; (*c*) reticulation in a mature cell.

6. Erythrocytes of the cunner showing, (*a* and *b*) primary granules and mitochondria; (*c* and *d*) reticulation in mature cells.

7. Erythrocytes of the scup showing, (*a* and *c*) primary granules and mitochondria; (*b*) reticulation in a mature cell; (*d*) reticulation in an immature cell.

8. Erythrocytes of the butterfish showing, (*a*) reticulation in a mature cell; (*b*) primary granules and mitochondria; (*c* and *d*) reticulation in immature cells.

9. Erythrocytes of the sand dab showing, (*a*) primary granules and mitochondria; (*b*) granules and reticulation; (*c* and *d*) induction of granules after twenty minutes.

10. Erythrocytes of the menhaden showing, (*a*) perinuclear primary granules and mitochondria; (*b*) granules after twenty minutes; (*c*) reticulation in a mature cell; (*d*) reticulation in an immature cell.

11. Erythrocytes of *Cyprinodon* showing, (*a* and *b*) primary granules and granular mitochondria; (*c* and *d*) reticulation in mature cells.

12. Erythrocytes of *Fundulus majalis* showing, (*a*) induction of granules after twenty minutes; (*b* and *c*) primary granules and mitochondria; (*d*) reticulation in a mature cell.

13. Erythrocytes of the mackerel showing, (*a*) reticulation in an immature cell; (*b*) perinuclear primary granules and mitochondria; (*c*) induction of granules after twenty minutes; (*d*) reticulation in a mature cell.

14. Erythrocytes of the alewife showing, (*a*) induction of granules after twenty minutes; (*b*) reticulation in a mature cell; (*c*) primary granules and mitochondria; (*d*) reticulation in an immature cell.

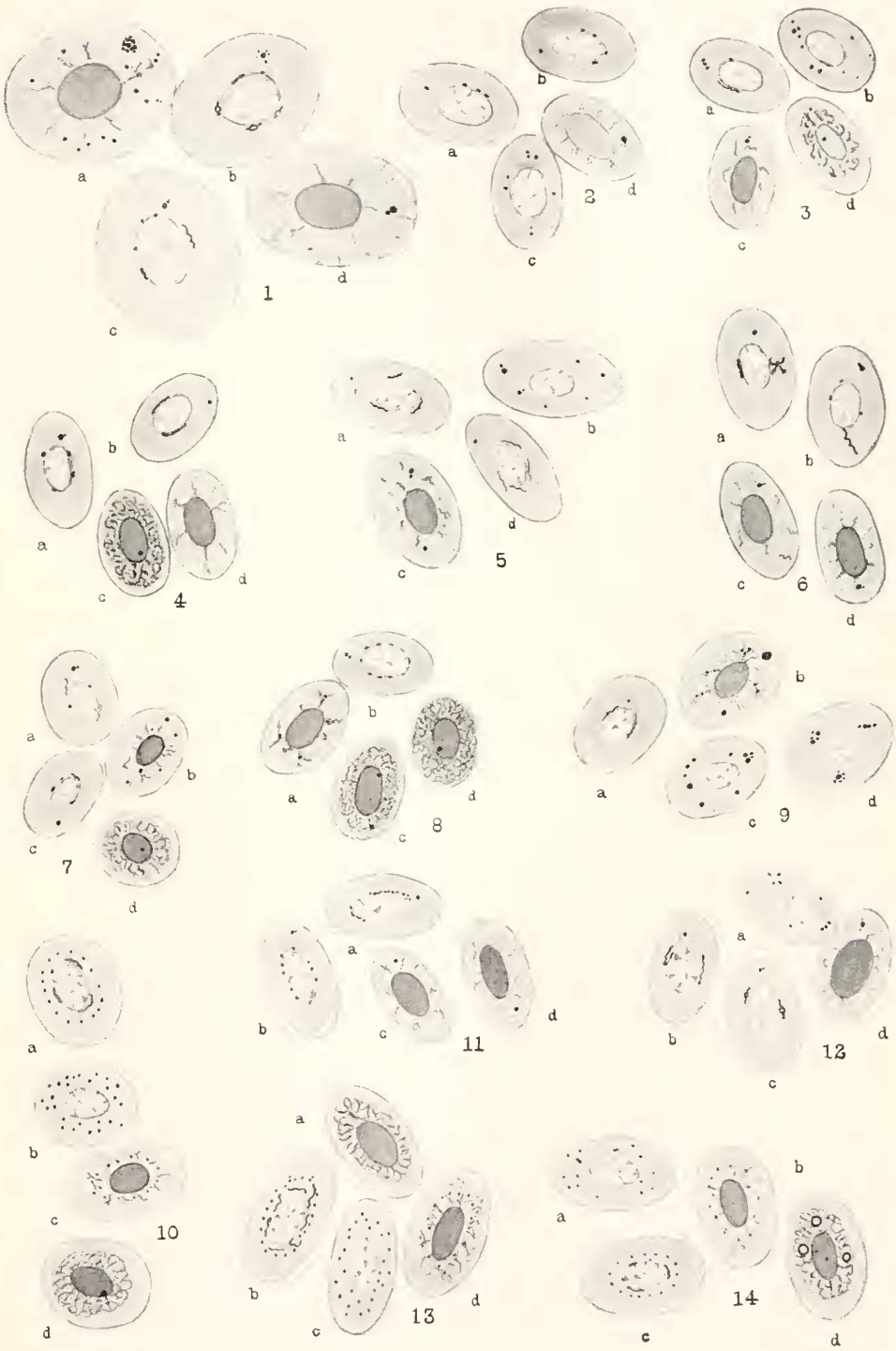
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Plate II

Explanation of Figures

15. Erythrocytes of the smooth dogfish showing, (*a*) the primary granules and mitochondria; (*b*) reticulation in a mature cell; (*c*) reticulation in an immature cell.

16. An erythrocyte of *Triturus viridescens* showing reticulation of a mature cell. Note the complete absence of granules.

17. Erythrocytes of the spotted skate showing, (*a*, *b*, and *c*) primary granules and mitochondria; (*d*) reticulation in a mature cell.

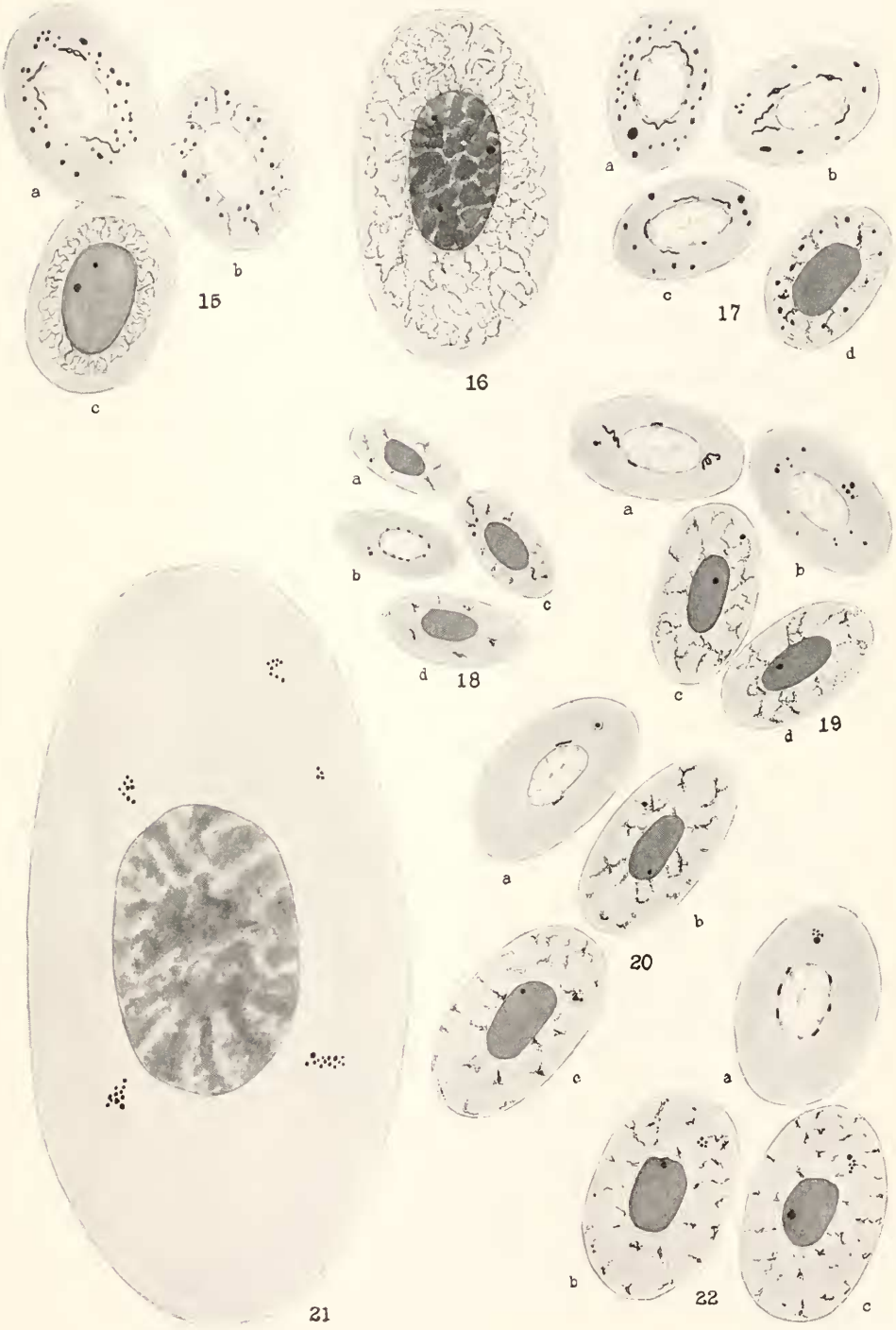
18. Erythrocytes of the domestic fowl showing, (*a*, *c*, and *d*) reticulation of mature cells; (*b*) primary granules and mitochondria.

19. Erythrocytes of the fence lizard showing, (*a*) primary granules and mitochondria; (*b*) induction of granules after twenty minutes; (*c* and *d*) reticulation and nucleoli in mature cells.

20. Erythrocytes of the horned toad showing, (*a*) primary granule and mitochondria; (*b* and *c*) reticulation and nucleoli in mature cells.

21. An erythrocyte of *Amphiuma means* from a smear stained by Wright's method, showing the primary granules as clusters of basophilic bodies.

22. Erythrocytes of the painted turtle showing, (*a*) the primary granules and mitochondria; (*b* and *c*) reticulation and nucleoli in mature cells.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